# OBSERVATIONS ON THE GROWTH OF DUNALIELLA EUCHLORA IN CULTURE[1]

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Under natural conditions, an intimate, but poorly understood relationship exists between bacteria and phytoplankton. Some investigators have claimed that phytoplankton produce a substance which is inhibitory to the growth of bacteria (Steemann Nielsen, 1955a, 1955b; Steemann Nielsen and Jensen, 1957). Contrariwise, the results of Waksman *et al.* (1937) suggest a harmonious relationship between algae and bacteria.

In experimental work, the use of bacteria-free cultures is customary. It has not always been possible to obtain bacteria-free cultures, and the assumption has been that the growth of the alga would have not been altered if axenic cultures had been used (*cf.* Goldberg *et al.,* 1951; Kain and Fogg, 1958). This assumption seems correct when using media with no organic additions (McLachlan, unpublished). However, little is known of the effects of bacteria on the growth of algae in cultures containing organic supplements.

In the present investigation, growth of the green flagellate *Dunaliella cuchlora* WHOI-1 in pure culture and contaminated cultures containing organic enrichments was studied. Growth was estimated by cell numbers and chlorophyll *a* synthesis. The production of algal and bacterial inhibitors was also investigated.

## Materials and Methods

A pure culture of *Dunaliella cuchlora* Lerche strain WHOI-1 (McLachlan, 1959) was used in this study. The alga was grown in a modification of the ASP medium of Provasoli *et al.* (1957); the composition of this medium is presented in Table I. In some of the experiments, the ASP medium was enriched with organic material by the addition of beef extract (0.3 g./l.) and bactopeptone (0.5 g./l.). At 16% nitrogen, this corresponds to an addition of about 9.1 mM of organic nitrogen, or nine times as much as is available in the basic medium. The cultures were incubated at 16° C. under 3,000 meter-candles of illumination provided by 40-watt fluorescent lights. Growth of the alga was determined by cell counts made in duplicate with a total of eight replicate counts using a Levy hemocytometer, and is expressed as $\log_2 N_t/N_o$ where $N_o$ is the concentration of cells at inoculation and $N_t$ the concentration at time t.

Chlorophyll *a* was measured spectrophotometrically in acetone extractions according to the Richards with Thompson method (1952) as modified for use with the millipore membrane filter procedure of Creitz and Richards (1955). The con-

[1] Contribution no. 1010 from the Woods Hole Oceanographic Institution. This investigation was supported, in part, by a grant from the National Science Foundation.

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TABLE I

*Composition of the modified ASP medium*

| | | | |
|---|---|---|---|
| NaCl | 410 mM | $FeCl_3 \cdot 6H_2O$ | 1.5 μM |
| $MgSO_4 \cdot 7H_2O$ | 24 mM | $H_3BO_3$ | 185.0 μM |
| $MgCl_2 \cdot 6H_2O$ | 22 mM | $MnCl_2 \cdot 4H_2O$ | 7.0 μM |
| $CaCl_2 \cdot 2H_2O$ | 10 mM | $ZnCl_2$ | 0.8 μM |
| $KNO_3$ | 1 mM | $CoCl_2 \cdot 6H_2O$ | 0.02 μM |
| $K_2HPO_4$ | 100 μM | $CuCl_2 \cdot 2H_2O$ | 0.0002 μM |
| $Na_2SiO_3 \cdot 9H_2O$ | 100 μM | $Na_2EDTA$ | 30.0 μM |

centration of chlorophyll *a* was determined using the nomographs of Duxbury and Yentsch (1956), and the organic nitrogen content of the cells was estimated by the procedure of Yentsch and Vaccaro (1958).

## RESULTS

1. *Growth in contaminated beef extract-peptone cultures.* To determine the effect of bacterial contamination on the growth of *Dunaliella euchlora,* the alga was inoculated into autoclaved and unautoclaved ASP medium, and autoclaved and

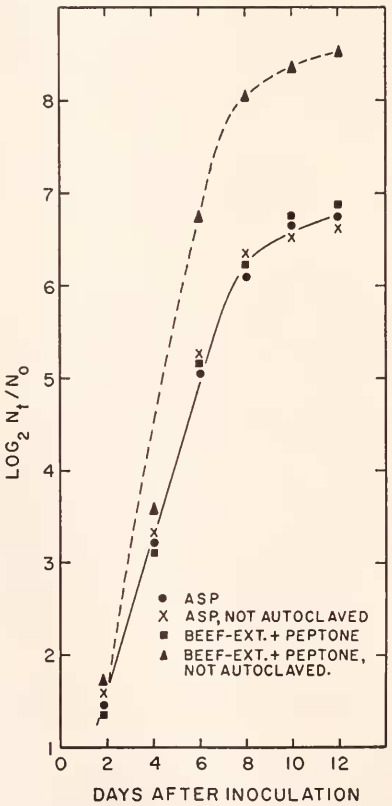


FIGURE 1. Growth of the alga in contaminated and non-contaminated ASP medium and contaminated and non-contaminated ASP medium containing beef extract and peptone.

unautoclaved ASP cultures containing beef extract and peptone. In the unautoclaved cultures with organic enrichments, approximately 2.5 times as many cells were obtained as in the other cultures (Fig. 1). The growth rate and the final number of cells in the other treatments were approximately the same. From these results it can be seen that the addition of beef extract and peptone *per se* did not increase the growth of the alga, but bacterial breakdown of these materials was of considerable benefit.

The unautoclaved cultures with organic additions became very dense, and after twelve days of growth contained approximately three times as much chlorophyll *a*

TABLE II

*The size of the population of* Dunaliella euchlora, *and the chlorophyll* a *content after growth in media treated as discussed in the text*

| Experiment | Treatment | Days of growth | No. cells/ml. $\times 10^4$ | Mg Chl$^a$/cell $\times 10^{-9}$ |
|---|---|---|---|---|
| Beef extract-peptone no. 1 | Enriched and contaminated | 12 | 1,089 | 2.24 |
| | Enriched, not contaminated | 12 | 397 | 0.74 |
| | ASP-autoclaved | 12 | 384 | — |
| | ASP-not autoclaved | 12 | 370 | — |
| Nitrate-nitrogen | 1.0 mM | 14 | 444 | 0.41 |
| | 2.0 mM | 14 | 868 | 0.40 |
| | 5.0 mM | 14 | 839 | 0.44 |
| | 8.0 mM | 14 | 716 | 0.39 |
| | 10.0 mM | 14 | 617 | 0.51 |
| Beef extract-peptone no. 2 | −3 | 15 | 320 | 0.81 |
| | 0 | 15 | 634 | 0.66 |
| | +2 | 15 | 924 | 0.47 |
| | +4 | 15 | 1,631 | 0.60 |
| | +7 | 15 | 1,198 | 1.11 |
| | +11 | 15 | 792 | 0.68 |
| | Control | 15 | 497 | 0.79 |
| *Dunaliella* filtrate | ASP | 11 | 504 | 0.44 |
| | Autoclaved filtrate | 11 | 527 | 0.93 |
| | Unautoclaved filtrate | 11 | 491 | 1.27 |
| Bacteria filtrate | ASP | 12 | 477 | 0.43 |
| | Filtrate | 12 | 958 | 0.80 |

per cell as the autoclaved enriched culture (Table II). Using the method of Yentsch and Vaccaro (1958), it was found that in the enriched contaminated cultures about 10 mM per liter of organic nitrogen was incorporated into the algal cells, or approximately $0.91 \times 10^{-6}$ $\mu$M of nitrogen per cell. In contrast, the non-contaminated enriched culture contained about 0.9 mM per liter of organic nitrogen as algal cellular material, or approximately $0.23 \times 10^{-6}$ $\mu$M of nitrogen per cell. The chlorophyll content of the two cultures containing only inorganic additions was not determined, but other estimates have indicated that at the end of exponential growth, all of the added nitrate-nitrogen is organically incorporated. This suggests

that in the non-contaminated beef extract peptone culture all of the incorporated organic nitrogen had been obtained from the added nitrate-nitrogen, and not from the organic material.

2. *Growth in various concentrations of nitrate-nitrogen.* To determine if the number of cells and the amount of chlorophyll *a* obtained in the contaminated beef extract-peptone cultures could be obtained by the addition of inorganic nitrogen, sodium nitrate was added at the following concentrations: 1.0 (ASP level), 2.0, 5.0, 8.0 and 10.0 mM. The ASP concentration of potassium was maintained by the addition of potassium chloride, and the phosphorus concentration was maintained at 100 $\mu$M in all cultures. The two highest concentrations of nitrogen slightly inhibited the growth of the alga (Fig. 2), but the total number of cells in all cultures

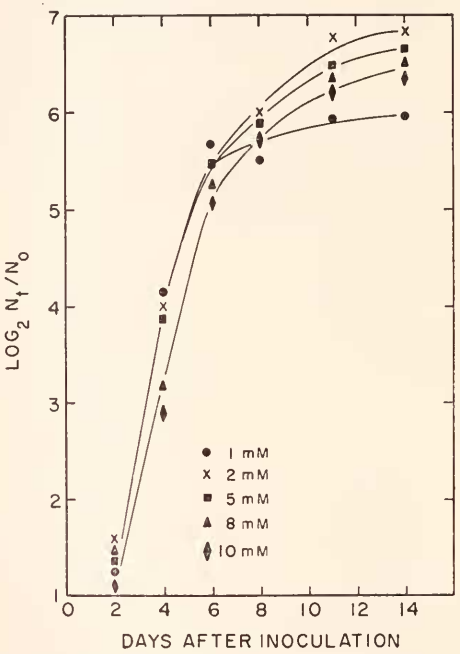


FIGURE 2. Growth of the alga in various concentrations of nitrate-nitrogen.

exceeded that of the 1.0 mM culture after fourteen days of growth. The maximum density of cells developed in the 2.0 and 5.0 mM cultures had a final cell count of almost twice that of the 1.0 mM culture (Table II), although the initial growth rates of these three cultures were approximately the same.

Pigment analysis showed that the chlorophyll *a* content of the cells in all cultures was about the same (Table II). The total number of cells obtained in the cultures containing 2.0 and 5.0 mM of nitrate-nitrogen approached that obtained in the contaminated beef extract-peptone culture of the previous experiment, but the amount of chlorophyll *a* was considerably less. Since the maximum amount of chlorophyll synthesized in these cultures was considerably less than was found in the contaminated culture containing beef extract and peptone, it may be concluded

that available nitrogen alone is not the limiting factor. Perhaps release of other nutrients in beef extract and peptone by bacterial activity or direct utilization of organic breakdown products by the alga also stimulated chlorophyll synthesis.

3. *Growth in beef extract-peptone cultures with periodic addition of bacteria.* To determine the effects of organic breakdown products on the growth of the alga at different phases of growth, a mixed culture of marine bacteria was obtained from the surface waters of Woods Hole Harbor, and uniform inocula were added periodically to cultures of *Dunaliella* containing beef extract and peptone. The original bacterial inoculum was collected using a sterile container so only bacteria present

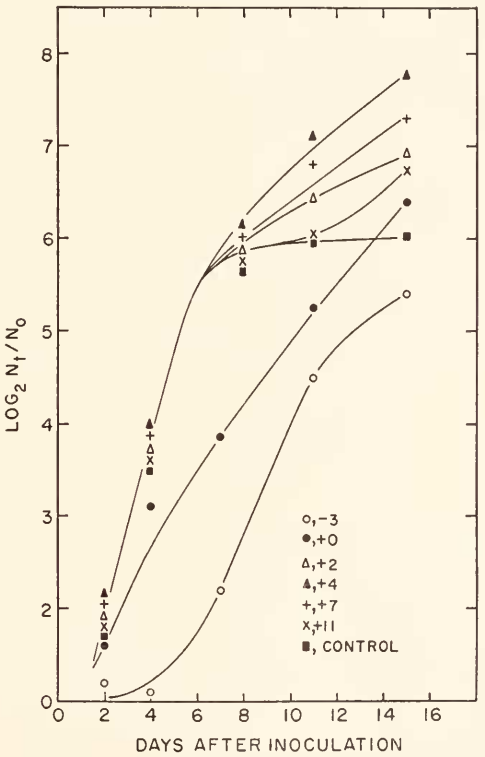


FIGURE 3. Growth of the alga in the ASP medium containing beef extract and peptone to which bacteria were added periodically.

in the surface water were introduced into culture. The bacteria were grown in the ASP medium with the addition of beef extract and peptone under the same conditions as the algal cultures. Before the bacterial cultures were used in the experimental work, they were carried through a number of transfers in order to obtain a uniform population.

Beef extract and peptone were added to the ASP medium and inoculated periodically with 1 ml. of the bacterial culture. In all cases the bacteria inocula were from three-day-old cultures when introduced into the experimental flasks. Inoculation

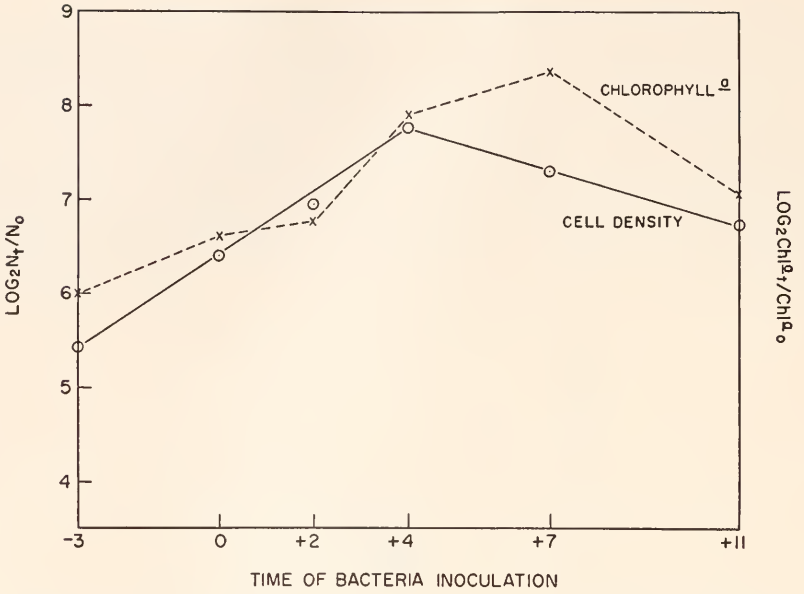


FIGURE 4. A comparison of algal cell density and chlorophyll *a* in cultures to which bacteria were added periodically.

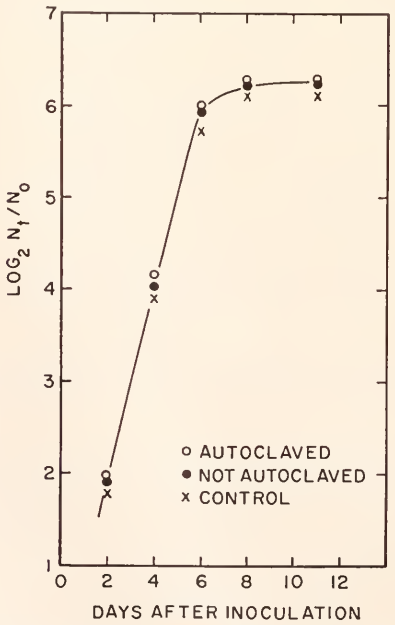


FIGURE 5. Growth of the alga in autoclaved and non-autoclaved algal filtrate.

took place at the following times: three days before inoculation with the alga, at the same time as the alga, two days, four days, seven days, and eleven days after inoculation with the alga.

Bacteria introduced three days before and at the time of inoculation with *Dunaliella* inhibited the growth of the alga (Fig. 3). In all cultures, except the one inoculated with bacteria three days before the alga, growth after fifteen days exceeded that of the control. In Figure 4 cell density and chlorophyll *a* after fifteen days of growth are presented. The greatest number of cells occurred in the culture which was inoculated with bacteria four days after the alga, and those cultures which were inoculated with bacteria prior to this time showed less growth. Less growth was also obtained in the cultures which had been inoculated with

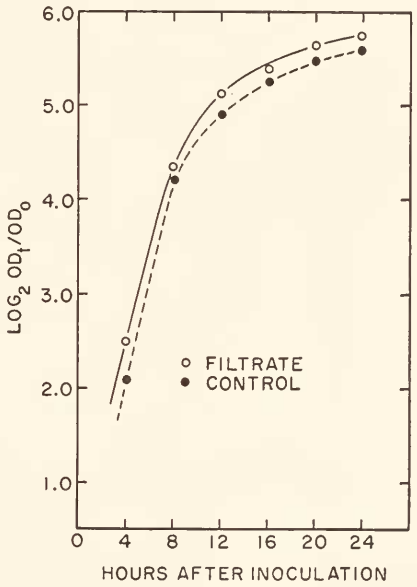


FIGURE 6. Growth of Woods Hole Harbor bacteria in the algal filtrate containing beef extract and peptone.

bacteria seven and eleven days after inoculation with the alga. However, the cells in the culture inoculated with bacteria seven days after the alga contained more chlorophyll *a* than the other cultures after fifteen days of growth (Table II). These results show that growth of the alga was directly related to the time at which the bacteria were added. Under the conditions of this experiment, chlorophyll *a* synthesis preceded cell division (Fig. 4). The highest concentration of chlorophyll per cell, however, was not as great as that obtained in the previous beef extract-peptone experiment.

4. *Growth of the alga in the algal filtrate.* To determine if auto-inhibitors are produced by *Dunaliella euchlora,* growth of the alga in the ASP medium was compared with growth in the filtrate of a *Dunaliella* culture which had reached maximum density. Part of the filtrate was autoclaved and the other part was not

autoclaved as there has been a suggestion of heat-labile inhibitors (Lefèvre *et al.*, 1952). Nitrogen, phosphorus, and iron were added to all cultures at ASP concentrations. Growth in all cultures was identical as shown by the data in Figure 5. The chlorophyll *a* content per cell was greater in the filtrate cultures than in the control whether or not the filtrate had been autoclaved (Table II). This indicates that the filtrate contained something which enhanced chlorophyll synthesis, but did not promote cell division.

5. *Growth of the bacteria in the algal filtrate.* To determine if *Dunaliella* filtrate would inhibit the growth of bacteria, a filtrate was obtained from a five-day

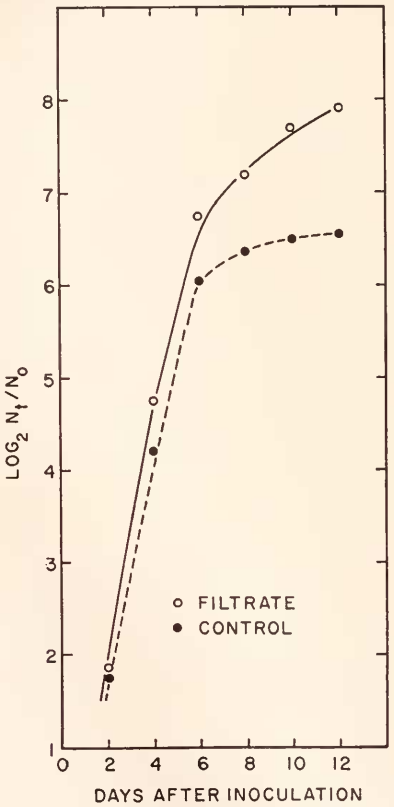


FIGURE 7. Growth of the alga in the Woods Hole Harbor bacteria filtrate.

culture of the alga. Beef extract and peptone were added to the filtrate and to the control ASP medium, and inoculated with the previously isolated Woods Hole Harbor bacteria. Growth of the bacteria was measured by optical density at 750 mμ using a Beckman model DU spectrophotometer, and is expressed as $\log_2 OD_t/OD_0$. The growth of the bacteria was approximately the same in the filtrate as in the enriched ASP medium with no suggestion of a bacteriostatic substance produced by the alga (Fig. 6).

6. *Growth of the alga in the bacterial filtrate.* To determine the effects of the bacterial filtrate on the growth of *Dunaliella,* a culture of the Woods Hole Harbor bacteria was grown in the ASP medium containing beef extract and peptone. After three days of growth, the culture was passed through a HA millipore filter resulting in a clear filtrate which was autoclaved and inoculated with the alga. There was a considerable increase in the maximum cell density in the filtrate culture as compared with the control (Fig. 7). The inhibition noted in the previous beef extract-peptone experiment where the bacteria had been added to the culture three days before the alga did not occur in this experiment. This suggests that the inhibitory substance is volatile or heat-labile. Pigment analysis also showed the cells in the filtrate culture contained considerably more chlorophyll *a* than those in the control culture (Table II).

## Discussion

The addition of beef extract and peptone to bacteria-free cultures of *Dunaliella euchlora* did not stimulate the growth of the alga. In the presence of bacteria, however, a considerable increase in the algal population, as determined by cell counts and chlorophyll analysis, was observed. This increase presumably resulted from the bacterial hydrolysis of the added organic material. The organic additions were not entirely inert to algal metabolism as the amount of chlorophyll *a* per cell in the uncontaminated cultures always exceeded that obtained with only the addition of inorganic salts.

The large amount of synthesis of chlorophyll *a* suggested that the alga was able to utilize nitrogenous breakdown products of the peptone and beef extract. A large fraction of the utilizable nitrogen from the beef extract and peptone was probably present as ammonia, nitrite, or simple organic compounds since the conversion of these to nitrate-nitrogen by marine bacteria is a slow process (Harvey, 1955; Sverdrup *et al.,* 1942). Harvey (1940) and Gibor (1956) observed that several species of *Dunaliella* were able to utilize some amino acids as nitrogen sources. There are also numerous reports of other autotrophic algae capable of utilizing organic nitrogen compounds (Fogg, 1953; Ryther, 1954). In addition, Huzisige *et al.* (1957) found that *Euglena* synthesized chlorophyll more rapidly when provided with casein hydrolysate than when provided with inorganic nitrogen compounds.

Growth of the alga in cultures containing organic additions was inhibited if bacteria were introduced before the alga began to grow. However, no inhibition of the alga was observed in the bacterial filtrate which had been autoclaved. This suggests a volatile or heat-labile inhibitor. Inhibition could have resulted from ammonia which is known to be toxic at low concentrations (Fogg, 1953; Myers, 1951). Any ammonia present in the bacterial filtrate would have been lost during autoclaving, and the inhibition noted in the second beef extract-peptone experiment may have been due to toxic concentrations of ammonia.

In the cultures which developed a heavy suspension of cells and chlorophyll, light and probably carbon dioxide may have been limiting, but organic carbon could have been utilized by the alga. Organic carbon could have been incorporated by the alga simultaneously with the utilization of organic nitrogen (Fogg, 1953; Krauss, 1958), or as separate compounds. If the alga is capable of heterotrophic growth, light would not necessarily be a limiting factor. It seems probable that the very

dense cultures obtained in this study may have been due in part to heterotrophic growth.

It was not possible in this study to demonstrate that *Dunaliella* inhibited the growth of bacteria, nor was there any indication of auto-inhibitors produced by the alga. The production of antibiotics by algae may not be a general phenomenon.

The authors wish to express their grateful appreciation to Dr. Bostwick H. Ketchum for his many helpful suggestions in preparing the manuscript.

## Summary

1. Bacteria added to cultures of *Dunaliella euchlora* in the presence of nitrogenous organic matter stimulated the growth of the alga, and enhanced the synthesis of chlorophyll *a*.

2. It was not possible to obtain comparable concentrations of chlorophyll *a* by the addition of nitrate-nitrogen, although a comparable number of cells could be obtained.

3. If the bacteria obtained a "head-start" in the enriched cultures, growth of the alga was inhibited. This inhibition could be overcome by autoclaving the filtrate from the bacteria cultures.

4. Growth of the bacteria was not inhibited in the algal filtrate, nor was the growth of the alga inhibited in the algal filtrate.

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